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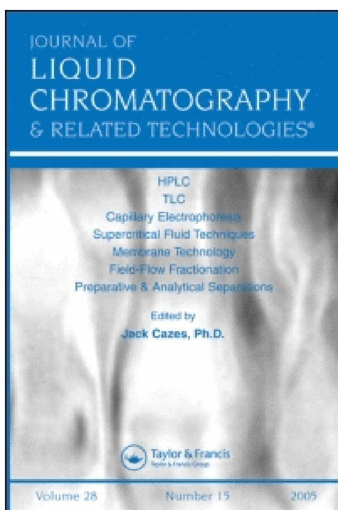

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High-Speed Gel Permeation Chromatography of Human Thyroglobulin and Sheep Liver Aldehyde Dehydrogenase

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HIGH-SPEED GEL PERMEATION CHROMATOGRAPHY OF HUMAN THYROGLOBULIN AND SHEEP LIVER ALDEHYDE DEHYDROGENASE.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF AMINO ACIDS, PEPTIDES AND PROTEINS XXII. PART XXI. REF.15.

ABSTRACT

This paper reports the application of high speed gel permeation techniques for the fractionation of human thyroglobulin preparations on chemically bonded, polar phase microparticulate silicas. Both resolution and recovery were found to be dependent on the ionic strength and pH of the eluent. At high salt concentrations significant hydrophobic interactions may occur between the support matrix and the protein solutes. The sheep liver enzyme, aldehyde dehydrogenase, was found to exhibit similar salt- and pH-dependent effects under these chromatographic conditions.

INTRODUCTION

Gel permeation chromatography has been very extensively used in studies on the isolation and structural characterisation of proteins and polypeptides. Because solutes are

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notionally separated on the basis of a non-ionic molecular size effect, this method also permits the molecular weight and radius of gyration of a protein to be determined under native or denatured conditions. A large variety of soft gel matrices, based on agarose, polydextran, polyacrylamide and its co-polymers, are now available with pore sizes compatible with the range of molecular weights (10^3-10^6 daltons and above) which polypeptides and proteins exhibit. Most of these gels, however, cannot withstand high pressures and they also undergo deformation with changes in experimental conditions (mobile phase composition, ionic strength, temperature, etc.) As a consequence, conventional size exclusion gels have proved unsuitable when used in conjunction with techniques usually associated with high performance liquid chromatography (HPLC). Recently, attention has been focused on the use of porous, surface-modified, silica packing materials for the high speed gel filtration of polypeptides and proteins. A variety of studies[1-5] have described the use of silica-based supports for analytical applications, eg. determination of molecular weight of polypeptides in the presence of dissociating reagents such as 0.1% sodium dodecylsulphate, 6M guanidine hydrochloride or 6M urea. At present, only limited information is available on the use of bonded polar phase silicas for the preparative chromatography of native proteins. Encouraging results have been reported[6,7] by several groups on the small scale separation of proteins, such as chymotrypsinogen A and catalase, without loss of biological activity under static or dynamic conditions. In this paper we report the group separation of human thyroid proteins with the μ Bondagel E-linear type packing material, on a 10μm porous silica matirx, which has been surface coated with a permanently bonded ether phase and with a reported nominal molecular weight separation range of 2 x 10^3-2 x 10^6 daltons under elution conditions which allow excellent recovery of

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human thyroglobulin. In addition, similar methods were applied to monitor the purification of sheep liver aldehyde dehydrogenase.

EXPERIMENTAL

High Performance Liquid Chromatography.

A Waters Assoc. (Milford, Mass., U.S.A.) HPLC system was used which consisted of a M6000A solvent delivery unit, a U6K universal liquid chromatograph injector and a M450 variable wavelength UV monitor coupled to a Rikadenki double channel chart recorder. The μBondagel E-linear columns (10μm, 30cm x 3.9mm ID), obtained from Waters Assoc., were coupled in series via zero dead volume connectors. The nominal void volume and inner volume were determined with blue dextran and L-tryptophan or β-mercaptoethanol respectively. The sample injections (10-50μl), at protein concentrations ranging from 1-20mg/mL, were made with Pressure-Lok liquid syringes from Precision Sampling Corp. (Baton Rouge, La., U.S.A.). Eluants were filtered and degassed prior to use as reported previously[8]. All chromatography was carried out at room temperature (ca.18°).

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Preparation of Protein Samples.

Crude human thyroglobulin was extracted from human thyroids by the method of Salvatore et al.[9]. Purified human thyroglobulin was prepared from the above extract by chromatography on Sepharose CL-4B using a 150mM NaCl-10mM Tris-HCl, pH 8.0, buffer followed by affinity chromatography using established procedures[10,11]. Antisera against human thyroid extracts and Sephadex G200 chromatographed human thyroglobulin were raised in guinea pigs as reported previously[10].

The crude sheep liver aldehyde dehydrogenase preparations were chromatographed on a Sephacryl S-200 column

(90 x 2cm) using a 25mM KH_2PO_4-0.1% β-mercaptoethanol, pH 7.3, buffer at a flow rate of 0.7ml/min. Enzymatic activity was determined spectrophotometrically using 100μl aliquots of each fraction and the propionaldehyde-NAD assay procedure[12].

RESULTS AND DISCUSSION

The potential of porous silica-based packing materials for the high speed chromatographic separation of proteins has been appreciated for a number of years, although until the advent of silicas with surface-bonded hydrophilic phases, their widespread application was not practical. In general, proteins are strongly adsorbed to silica matrices due to hydrogen bonding of the -NH or -OH groups of the biomolecule to oxygens of the siloxane chains and ionic interactions of solute cationic groups with the non-bridging silanol groups of the matrix. The hydrogen bonding, but not ionic, effects can be largely overcome by coating the silica with a polar phase. Schecter has used[12] this approach for the separation of several proteins on Carbowax-20m deactivated silicas. With the development of microparticulate packings made from porous silicas or glasses with various hydrophilic carbonaceous phases chemically bonded to the matrix surface, many of the limitations, including poor peak shape and low recoveries arising from the strong coulombic interactions between the support and the protein solutes have been largely overcome. It is now clear from a number of studies[1,4,6] on the elution characteristics of a group of similar globular proteins, including ovalbumin and bovine serum albumin, that the total permeation times obtained with these bonded silica-based size exclusion supports are considerably shorter than those observed with conventional polysaccharide gels of comparable pore size. Furthermore, the generally lower permeabilities of the microparticulate silica-based supports, compared to those of the conventional

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carbohydrate support, can be experimentally circumvented by increasing the column efficiencies, eg. by the use of packing materials with a narrow particle size distribution and appropriate pore diameter range, by optimising the flow rate and by taking advantage of solute-eluent interactions. Resolution and the extent of inclusion of protein solutes with a silica-based support of nominal particle size and pore diameter range should, however, respond to changes, inter alia, in the composition and the pH of the eluent in a similar predictable manner to that experienced with conventional gels. Both of the above eluent parameters are known to significantly affect the degree of hydration and the tertiary structure of proteins and they may also influence the pore structure ie. by changing the effective pore volume, of the packing material.

Thyroglobulin is the major glycoprotein of the thyroid gland and has usually been fractionated from the other thyroid proteins by agarose gel chromatography eg. on Sepharose CL-4B[10]. In preliminary experiments designed to test the suitability of columns packed with μBondagel E-linear to separate human thyroglobulin from the other thyroid proteins (total molecular weight range ca 3×10^4-7×10^5 daltons) present in crude extracts, the effects of eluent composition, pH and flow rate were examined. Flow rates up to 2.0mL/min gave reproducible elution profiles without significant changes in the elution volumes of the protein components with slightly improved resolution at the lower flow rates, eg. 0.5mL/min. The extent of peak inclusion, peak shape and recoveries of these thyroid proteins were, however, significantly affected by changes in the composition and pH of the eluent. For example, with low ionic strength buffers, eg. 5mM sodium acetate pH 6.9, up to 50mM ammonium acetate pH 5.5, or with chaotropic eluents, eg. 5M urea, at flow rates up to 2mL/min,

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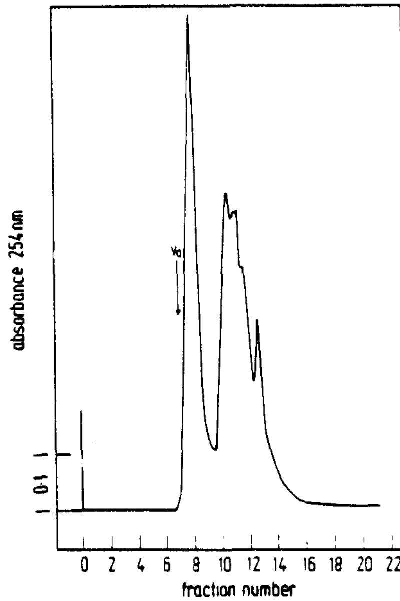

FIGURE (1a)

Gel exclusion chromatography of a crude human thyroid protein extract on dual μBondagel E-linear columns (2 x 30cm x 3.9mm I.D.) eluted with a 50mM ammonium acetate, pH 5.5, buffer at a flow rate of 2mL/min. The void volume (ca. 3.2mL) was calibrated with Blue Dextran 2000.

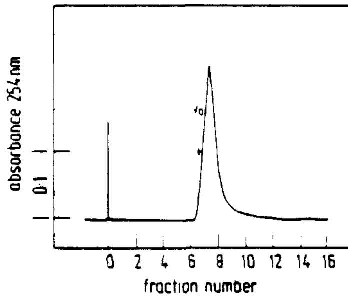

FIGURE (1b)

Gel exclusion chromatographic profile of human thyroglobulin previously fractionated on Sepharose CL-4B. Chromatographic conditions as in (1a).

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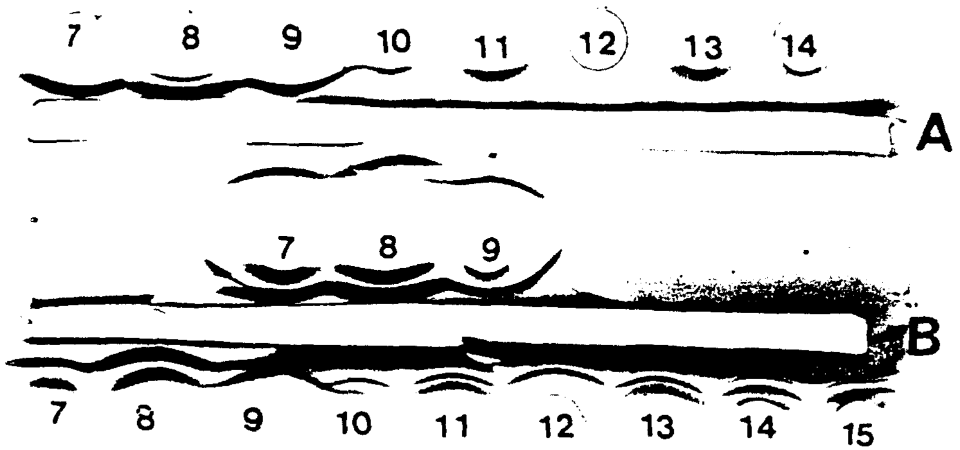

FIGURE (1c)

Immunodiffusion patterns for the fractions obtained from the crude human thyroid procein extract chromatographed as in (1a). Aliquots were diffused against anti-human thyroglobulin (A) and anti-human thyroid proteins (B) with well numbers corresponding to the chromatographic fraction numbers. Also shown are the immunodiffusion patterns of the recovered fraction 7-9 following lyophilisation.

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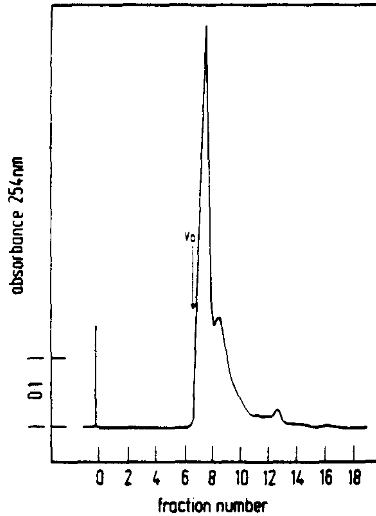

FIGURE (1d)

Gel exclusion chromatography of the crude human thyroid protein extract using conditions given in (1a) except that the pH of the eluent was increased to pH 6.9.

human thyroglobulin is essentially excluded despite the nominal 2×10^6 exclusion limit of the support, although high recoveries of the proteins, with preservation of antigenicity, are obtained with these conditions. Typical elution profiles obtained with a crude and purified human thyroglobulin preparation eluted with 50mM ammonium acetate pH 5.5, and the corresponding immunodiffusion patterns obtained with specific antisera are shown in Figure 1a-c respectively. The total time required for a single chromatographic run at a flow rate of 0.7mL/min was approximately 10 minutes. In spite of the relatively high flow rates and smaller column dimensions used in this study improved resolution is evident, compared to that obtained with soft gel supports[10]. As the ionic strength of the eluent was increased, eg. from 100mM to 1M sodium acetate, potassium

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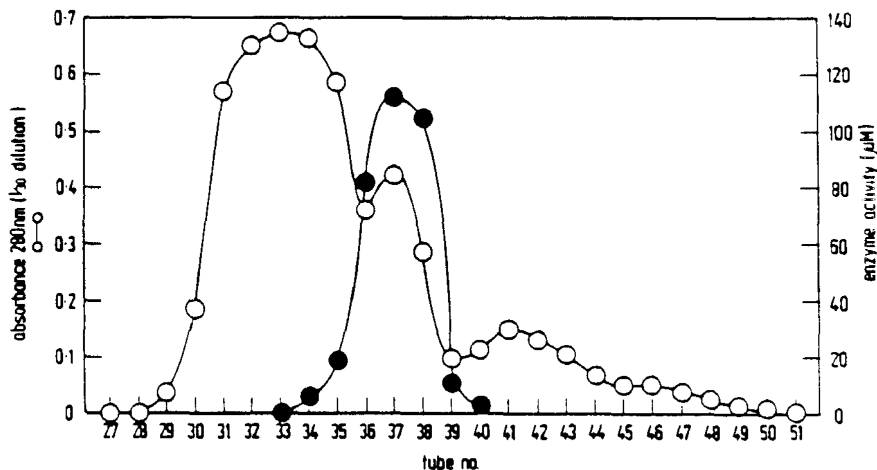

FIGURE (2a)

Gel filtration of a sheep liver aldehyde dehydrogenase preparation on a Sephacryl S-200 column (90 x 2.0cm), equilibrated in 25mM KH_2PO_4, pH 7.3 and 0.1% 2-mercaptoethanol at a flow rate of 0.7mL/min. The specific enzymatic activity was determined using the reported propionaldehyde-NAD assay procedure[12].

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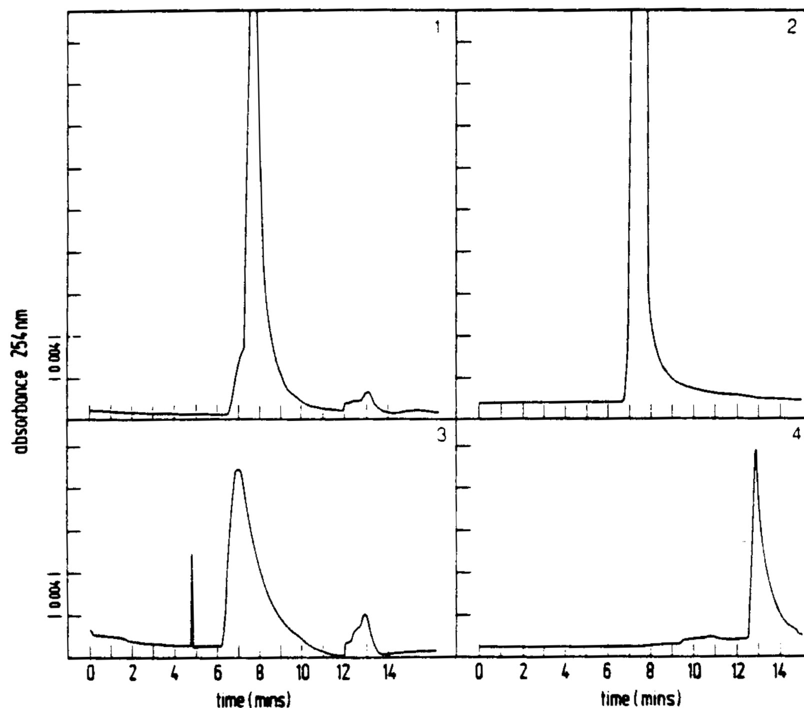

FIGURE (2b)

High speed gel exclusion chromatography of fractions from the Sephacryl S-200 separation of the sheep liver aldehyde dehydrogenase preparation. Chromatographic conditions: column, μBondagel E-linear (2 x 30cm x 3.9mm), flow rate 0.5mL/min., buffer, 5mM KH_2PO_4, pH 7.3. Under these chromatographic conditions, the recovery of the enzyme (V_e 3.8mL) was >88%. The figure shows profiles using aliquots from fraction 36 (1), dialysed fraction 38 (2) contaminant peak 34 (3) at pH 6.1 and fraction 37, containing β-mercaptoethanol (V_e 6.5mL), eluted at pH 5.1 (4).

dihydrogen phosphate etc., the inclusion volume of the thyroid proteins progressively increased. This progressive inclusion of human thyroglobulin and the other thyroid proteins presumably reflects changes in the radius of gyration of the macromolecule from an extended shape in lower molarity or chaotropic buffers to a more compact form in high ionic strength buffers. Furthermore, lower recoveries of these thyroid proteins were obtained with the higher ionic strength buffers, eg. for the crude thyroglobulin extract, 45% recovery of the proteins was obtained with 100mM potassium dihydrogen phosphate compared to >90% with 5M urea or 50mM ammonium acetate at pH 5.5. It is also noteworthy that both the recovery and resolution were very responsive to changes in pH. This effect can be seen by comparison of the chromatographic profiles (Figure 1a,d) obtained under identical conditions except for a pH change from pH 5.5 to pH 6.9. When the pH was lowered to value proximal to the iso-electric point of the thyroglobulin eg. in the range pH 4.5-5.1, the macromolecule could not be eluted.

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Sheep liver aldehyde dehydrogenase showed similar salt- and pH-dependent effects when chromatographed on μBondagel E-linear columns. Since good enzymatic recovery (ca. 88%) could be obtained with this support using a 5mM KH_2PO_4, pH 7.3, buffer it was possible to follow by HPLC the open column purification of this enzyme eg. on Sephacryl S-300 Superfine (Figure 2). The enzyme could not be eluted from μBondagel E-linear columns with the same buffer at pH 5.1 or with a 50mM KH_2PO_4, pH 7.3 buffer. The reduced recovery of this enzyme, as well as for human thyroglobulin noted above, at higher salt concentrations is indicative of the 'salting out' effect and suggests that significant hydrophobic interactions of these proteins may occur with the support matrix under these elution conditions. Oval-

bumin, and several other globulin proteins, were also found to exhibit a similar salt effect.

It may be concluded that, under appropriate elution conditions, μBondagel E-linear columns in addition to the related μBondagel E-125 Å to E-1000Å supports[4], can be used for the rapid, molecular size selective separations of proteins under analytical and semi-preparative conditions. In the present study, milligram quantities of human thyroglobulin could be rapidly fractionated from other thyroid proteins, or desalted, on standard (30cm x 3.9mm I.D.) columns with separation times approximately 100 times shorter than those of the carbohydrate type of gel support, without loss of resolution. Although their utility for large-scale preparative separations has yet to be determined, we have found that these, and related silica based chromatographic supports are very useful for the high speed gel permeation chromatography of proteins available in small quantities eg. the separation of thyroid membrane glycoprotein antigens. These results will be reported[14] in more detail elsewhere.

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